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(54) Title: ACID-LABILE SUBUNIT (ALS) OF INSULIN-LIKE-GROWTH FACTOR (IGF) BINDING PROTEIN COMPLEX

Gly
AspProGlyThrProGlyGluAlaGluGlyProAlaCysProAlaAlaCys- (I)
Ala

(57) Abstract

The acid-labile sub-unit (ALS) of insulin like growth factor binding protein complex in biologically pure form is described. ALS has a molecular weight between 80-115kd as determined by SDS polyacrylamide gel electrophoresis, run under reducing conditions; and a partial N-terminal amino acid sequence (I), wherein the first amino acid may be Gly or Ala. Also described are methods of producing ALS, compositions containing the in-vivo IGF protein complex, methods of detecting ALS in body fluids, recombinant nucleic acid sequences encoding ALS, and expression vectors and host cells containing such nucleic acid sequences.

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Acid-labile subunit (ALS) of Insulin-like-growth factor (IGF) binding protein complex

FIELD OF INVENTION

This invention relates to a previously unknown and uncharacterised polypeptide, hereinafter referred to as the acid-labile sub-unit (ALS) of insulin like growth factor (IGF) binding protein complex.

Peptides of the insulin-like growth factor (IGF) family resemble insulin both in their structure and in many of their actions. The IGF family consists of two members designated IGF-I and IGF-II (IGFs). The IGFs exhibit a broad spectrum of biological activity, including anabolic insulin-like actions (e.g. stimulation of amino acid transport and glycogen synthesis), mitogenic activity and the stimulation of cell differentiation.

Human IGF-I and IGF-II have been extensively characterized, and have been found to have molecular weight of approximately 7.6Kd (IGF-I) and 7.47Kd (IGF-II).

Unlike most peptide hormones, IGFs are found in the circulation (in-vivo) and in cell culture medium in association with one or more binding proteins. The nature of the binding protein or binding proteins associated with the IGFs has been the subject of debate. Wilkins, J.R. and D'Ercole, A.J. (1985, J. Clin. Invest. 75, 1350-1358) have proposed that the in-vivo form of IGF is a complex comprising IGF in association with six identical sub-units having a molecular weight of 24Kd to 28Kd. In a second proposal, the in-vivo form of IGF is said to be associated with an acid-stable binding protein and an acid-labile protein(s) to generate a complex of approximately 150Kd (Furlanetto, R.W. (1980) J.Clin. Endocrinol. Metab. 51, 12-19).

We have previously identified an acid-stable serum protein which has a single IGF-binding site per molecule, is immunologically related to the 150Kd in-vivo form of IGF and which has an apparent molecular weight of approximately 53Kd (Baxter, R.C., and Martin, J.L. (1986) J. Clin. Invest. 78, 1504-1512; and Martin, J.L. and Baxter, R.C. (1986) J. Biol. Chem. 261, 8754-8760). This 53Kd IGF binding protein (BP53) appears to correspond to the acid stable binding protein proposed by Furlanetto. The 53Kd protein is the highest molecular weight member of a family of acid-stable IGF binding proteins. Other members of this family have approximate molecular weights of 20, 34, 36, 30 and 47Kd, and collectively fall within the definition "acid-stable IGF binding protein".

We have now surprisingly identified an acid-labile protein, which when incubated with the 53Kd acid stable protein occupied by IGF converts it

to a high molecular weight complex, corresponding to the in-vivo form of IGF.

SUMMARY OF THE INVENTION

According to one aspect of the invention there is provided the acid-labile sub-unit (ALS) of insulin like growth factor binding protein complex in biologically pure form, which preferably has the following partial N-terminal amino acid sequence:

Gly
AspProGlyThrProGlyGluAlaGluGlyProAlaCysProAlaAlaCys-Ala

wherein the first amino acid may be Gly or Ala.

In another aspect of the invention there is provided a composition of matter consisting essentially of the acid-labile sub-unit (ALS) of the insulin like growth factor binding complex.

In another aspect of the invention there is provided a composition, reconstituted from three polypeptide components, namely, IGF, BP-53 and ALS. The composition may be formulated to be in association with one or more pharmaceutically acceptable carriers or excipients.

In yet another aspect of the invention there is provided a process for the preparation of ALS, which comprises the steps of:

(a) applying a source of ALS to a support matrix having attached thereto IGF bound to or associated with the acid-stable IGF binding protein, whereby the ALS in the applied material binds to the acid stable binding protein and non-bound material is separated from the support matrix; and

(b) selectively eluting and recovering the ALS protein from the IGF protein complex.

Preferably, ALS is prepared by a process comprising the steps of:

(a) binding IGF to a support matrix;

(b) adding the acid-stable IGF binding protein to the support matrix such that it binds to or is associated with the IGF;

(c) applying a source of ALS to the support matrix whereby the ALS in the applied material binds to the acid stable protein and non-bound material is separated from the support matrix;

(d) selectively eluting the ALS protein from the IGF protein complex; and

(e) optionally further fractionating the recovered ALS by HPLC or FPLC.

According to a further aspect of the invention there is provided a method for detecting the levels of ALS in body fluids, which comprises fractionating the body fluids on a size fractionation matrix to separate free ALS from the other components of the insulin growth factor binding complex, and thereafter quantitating the levels of ALS in the fractionated sample.

In still another aspect of the invention there is provided a recombinant nucleic acid sequence encoding the acid-labile sub-unit (ALS) of insulin like growth factor. The recombinant nucleic acid sequence preferably encodes a polypeptide having the following partial N-terminal amino acid sequence:

Gly
AspProGlyThrProGlyGluAlaGluGlyProAlaCysProAlaAlaCys-
Ala.

wherein the first amino acid is Gly or Ala.

The invention also relates to an expression vector containing a recombinant nucleic acid sequence

encoding ALS, host cells transformed with such a vector, and ALS when produced by such host cells.

In yet another aspect of the invention there are provided polypeptides comprising fragments of ALS, and nucleic acids comprising sequences encoding same, which include or encode residues 1-5, 2-7, 5-9, 7-11, 8-14, 11-15, 13-17, 3-9, 2-8, 4-10, 6-12, 8-14, 10-16, 12-18, 1-6, 3-9, 5-11, 7-13, 9-15, 11-17, 4-9, 6-11, 8-13, 10-15, or 12-17 of ALS.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to ALS, a polypeptide which binds to, and stabilizes in-vivo, a complex between IGF and its acid-stable binding protein BP-53. IGF can be IGF-I or IGF-II.

BP-53 is a glycoprotein, that is, one or more carbohydrate chains are associated with the BP-53 polypeptide sequence. Where mention is made of the acid-stable IGF binding protein or BP-53, it is to be understood to refer to an acid-stable protein capable of binding to insulin like growth factor, and capable of forming a complex with ALS and IGF. As long as the acid-stable IGF binding protein or BP-53 satisfies these functions, it may be non-glycosylated, partly glycosylated, modified by way of amino acid deletions or substitutions or insertions, and may have a molecular weight of 20, 30, 34, 36, 47 and 53Kd. The precise molecular weight of this component is generally unimportant.

In accordance with the present invention and using the methods disclosed herein, said ALS is biologically pure. By biologically pure is meant a composition comprising at least 65% by weight of ALS and preferably at least 75% by weight. Even more

preferably, the composition comprises at least 80% ALS. Accordingly, the composition may contain homogeneous ALS. In this specification, the term "biologically pure" has the same meaning as "essentially or substantially pure".

Where this invention relates to a composition of matter consisting essentially of ALS, the term "composition of matter" is to be considered in a broad context. The composition of matter may be ALS itself, or ALS in association with one or more pharmaceutically or veterinarially acceptable carriers or excipients. Suitable carriers may include water, glycerol, sucrose, buffers or other proteins such as albumin, etc. The term "consisting essentially of" has the same meaning as "biologically pure" discussed above.

By binding to IGF is meant the ability of ALS to bind to complexes formed when IGF is bound or associated with an acid-stable component, BP-53.

ALS is a glycoprotein, that is, one or more carbohydrate chains are associated with the ALS polypeptide sequence. This invention extends to ALS in its fully glycosylated, partially glycosylated or non-glycosylated forms, which may be readily prepared according to methods well known in the art. For example, ALS prepared according to the methods disclosed herein may be reacted with enzymes, such as endoglycosidases, to remove N-linked carbohydrate either partially or totally. O-linked carbohydrate may similarly be removed by well known methods.

As mentioned previously, ALS preferably has the following partial N-terminal amino acid sequence:-

Gly
AspProGlyThrProGlyGluAlaGluGlyProAlaCysProAlaAlaCys-
Ala

where the first amino acid may be Gly or Ala.

It is to be understood, however, that the ALS of the present invention is not restricted to possessing the above N-terminal amino acid sequence. Rather, ALS is functionally defined as an acid-labile polypeptide which is capable of binding to or associating with complexes formed when IGF is bound or associated with the acid stable binding protein BP-53 defined above. The definition ALS extends to encompass synthetic and naturally occurring amino acid substitutions, deletions and/or insertions to the natural sequence of ALS, as will be readily apparent to the skilled artisan. For example, genetic engineering means can be readily employed using known techniques to substitute, delete and/or insert amino acids.

Generally, and in no way limiting the invention, ALS may be characterized in that it:

(i) is acid-labile, that is, it is unstable at a pH less than 4,

(ii) binds to an acid stable IGF binding protein which is occupied by IGF, and

(iii) has an approximate molecular weight between 80Kd and 115Kd as determined by SDS-PAGE.

ALS referred to herein is human ALS. Animal ALS, which is capable of forming a complex with animal IGF, is also to be understood to fall within the scope of the term ALS.

ALS is contemplated herein to be useful in the preparation of the physiological IGF complex which comprises IGF, BP-53 and ALS. Such a complex may be

useful in wound-healing and associated therapies concerned with re-growth of tissue, such as connective tissue, skin and bone; in promoting body growth in humans and animals; and in stimulating other growth-related processes. The ALS protein also confers a considerable increase in the half-life of IGF in-vivo. The half-life of IGF per se, unaccompanied by binding proteins, is only a few minutes. When IGF is in the form of a complex with the acid-stable IGF binding protein, and the ALS protein, its half-life is increased to several hours, thus increasing the bio-availability of IGF with its attendant therapeutic actions. Furthermore, pure ALS may be used to raise specific monoclonal or polyclonal antibodies, in order to establish a radioimmunoassay or other assay for ALS. Measurement of ALS in human serum may be useful in diagnosing the growth hormone status of patients with growth disorders.

The IGF binding protein complex formed by admixing ALS, IGF and the acid stable protein BP-53, where each component is preferably in biologically pure form, may be formulated with suitable pharmaceutically and/or therapeutically or veterinarially acceptable carriers and used for example, in growth promotion or wound treatment in human and non-human animals. Examples of pharmaceutically acceptable carriers include physiological saline solutions, serum albumin, or plasma preparations. Depending on the mode of intended administration, compositions of the IGF binding protein complex may be in the form of solid, semi-solid or liquid dosage preparations, such as for example, tablets, pills, powders, capsules, liquids,

suspensions or the like. Alternatively, the IGF binding protein complex may be incorporated into a slow release implant, such as osmotic pumps for the release of material over an extended time period.

The amount of the IGF binding protein complex administered to human patients or animals for therapeutic purposes will depend upon the particular disorder or disease to be treated, the manner of administration, and the judgement of the prescribing physician or veterinarian.

ALS may be purified from human serum or plasma, or plasma fractions such as Cohn Fraction IV. Purification from whole serum is preferred, this being the most economical and plentiful source of material and giving the highest yield. Purification of ALS exploits the physiological interaction between IGF, BP-53 and ALS. ALS is recovered from human serum by passing the serum through a support matrix having IGF-BP-53 bound or associated therewith. Reference to association means a non-covalent interaction, such as electrostatic attraction or hydrophobic interactions. ALS bound to the IGF-BP-53 affinity matrix may then be eluted by disrupting the interaction between ALS and the affinity matrix, for example by increasing ionic strength (e.g. at least 0.3M NaCl, or other equivalent salt) or conditions of alkaline pH (above pH 8).

A source of ALS such as whole plasma or Cohn Fraction IV thereof may be fractionated on an ionic resin to enrich the amount of ALS prior to application to the affinity matrix. A cation exchange resin is preferred. Optionally, ALS purified by affinity chromatography is subjected to a further purification step such as HPLC or FPLC

(Trademark, Pharmacia). The HPLC step may, for example, be conducted using a reverse phase matrix, a gel permeation matrix or an ionic matrix.

Where this invention is concerned with antibodies which are capable of binding to ALS, the antibodies may be monoclonal or polyclonal. Such antibodies may be used to measure ALS levels in serum, and may form part of a diagnostic kit for testing growth related disorders. Antibodies against ALS may be prepared by immunizing animals (for example; mice, rats, goats, rabbits, horses, sheep or even man) with purified ALS according to conventional procedures (Goding, J.W. (1986) Monoclonal Antibodies: Principles and Practices, 2nd Edition, Academic Press). Serum proteins may, for example, be attached to a support matrix, and incubated with anti-ALS antibodies which may be labelled with reporter groups (for example, fluorescent groups, enzymes or colloidal groups) to detect ALS. Alternatively, non-labelled anti-ALS antibodies bound to ALS may be reacted with suitable agents (such as antibodies directed against anti-ALS antibodies or anti-immunoglobulin antibodies) to detect antibody binding, and thus quantitate ALS levels.

Where this invention relates to a recombinant nucleic acid molecule, said molecule is defined herein to be DNA or RNA, encoding ALS or parts thereof. In one embodiment, the recombinant nucleic acid molecule is complementary DNA (cDNA) encoding mammalian and preferably, human ALS, or parts thereof including any base deletion, insertion or substitution or any other alteration with respect to nucleotide sequence or chemical composition (e.g. methylation). ALS encoded by cDNA is herein referred

to as recombinant ALS.

A recombinant nucleic acid which exhibits at least 60% sequence homology or more preferably 80 to 99% homology with nucleic acid (cDNA, DNA, RNA) encoding ALS, or which encodes a protein having the biological activity of ALS, is to be regarded as nucleic acid encoding ALS.

Methods considered useful in obtaining recombinant ALS cDNA are contained in Maniatis et. al., 1982, in Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, New York, pp 1-545. Briefly, polyadenylated mRNA is obtained from an appropriate cell or tissue source, such as liver. Optionally, mRNA is fractionated on agarose gels, or gradient centrifugation, and translated and assayed for ALS, such as, for example, by immunoprecipitation. Enriched or unenriched mRNA is used as a template for cDNA synthesis. Libraries of cDNA clones are constructed in the PstI site of a vector such as pBR 322 (using homopolymeric tailing) or other vectors; or are constructed by ligating linkers (such as Eco R1 linkers) onto the ends of cDNA, which is then cloned into a vector having sites complementary to said linkers. Specific cDNA molecules in a vector in a library are then selected using specific oligonucleotides based on the aforementioned N-terminal amino acid sequence of ALS. Alternatively, commercially available human lambda libraries may be screened with oligonucleotides. In an alternative approach, the cDNA may be inserted into an expression vector such as lambda gt 11, with selection based on the reaction of expressed protein with a specific antibody raised against purified ALS. In any event, once identified,

cDNA molecules encoding all or part of ALS are then ligated into expression vectors. Additional genetic manipulation is routinely carried out to maximise expression of the cDNA in the particular host employed.

Accordingly, ALS is synthesized in vivo by inserting said cDNA sequence into an expression vector, transforming the resulting recombinant molecule into a suitable host and then culturing or growing the transformed host under conditions requisite for the synthesis of the molecule. The recombinant molecule defined herein should comprise a nucleic acid sequence encoding a desired polypeptide inserted downstream of a promoter functional in the desired host, a eukaryotic or prokaryotic replicon and a selectable marker such as one resistant to an antibiotic. The recombinant molecule may also require a signal sequence to facilitate transport of the synthesized polypeptide to the extracellular environment. Alternatively, the polypeptide may be retrieved by first lysing the host cell by a variety of techniques such as sonication, pressure disintegration or detergent treatment. Hosts contemplated in accordance with the present invention can be selected from the group comprising prokaryotes (e.g., Escherichia coli, Bacillus sp., Pseudomonas sp.) and eukaryotes (e.g., mammalian cells, yeast and fungal cultures, insect cells and plant cultures). The skilled person will also recognize that a given amino acid sequence can undergo deletions, substitutions and additions of nucleotides or triplet nucleotides (codons). Such variations are all considered within the scope of the present invention. Additionally, depending on the host

expressing recombinant ALS, said ALS may or may not be glycosylated. Generally, eukaryotic cells, for example mammalian cells and the like, will glycosylate the recombinant ALS. Prokaryotic cells, for example, bacteria such as Escherichia coli and the like, will not glycosylate the recombinant ALS. Both glycosylated and non-glycosylated ALS are encompassed by the present invention, as has been previously mentioned.

ABBREVIATIONS

IGF	-	insulin-like growth factor
SDS-PAGE	-	sodium dodecylsulphate polyacrylamide gel electrophoresis
Kd or K	-	kilodaltons
GH	-	growth hormone

The following drawings and Examples are illustrative of, but in no way limiting, on the present invention.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1: Affinity chromatography of ALS. A 132-ml pool of fractions partially purified by DEAE-Sephadex chromatography was loaded at 0.13 ml/min onto a 1 x 15 cm affinity column containing a mixture of IGF-I and IGF-II covalently bound to agarose, to which BP-53 had been noncovalently adsorbed. The column was washed with 150 ml of 50 mM Na phosphate, pH 6.5 (Wash #1) and 50 ml of 5 mM Na phosphate, 50 mM NaCl, pH 6.5 (wash #2), at 1 ml/min. ALS was eluted by applying 50 mM Tris-HCl, 0.3 M NaCl, pH 8.5, at 0.5 ml/min.

FIGURE 2: SDS-polyacrylamide gel

electrophoresis of purified ALS. Left panel: Untreated, acidified, and N-glycanase-treated samples (15 µg/lane) run under nonreducing conditions. Right panel: the same samples in reverse order, run under reducing conditions. Gels were stained with Coomassie blue. The molecular masses (in kDa) of standard proteins, shown in the right hand lane for the reduced gel, are also indicated by arrows on the left for standards run on the nonreduced gel.

FIGURE 3 shows fractionation of human serum on a column of DEAE-Sephadex A-50. One milliliter dialyzed serum was loaded onto a 1 x 17.5 cm. gel bed, the column was washed with 35 mL 0.05 mol/L Tris-HCl. pH 8.2 and elution commenced with 50 mL of the same buffer containing 0.15 mol/L NaCl. Elution was then continued with the same buffer containing 0.6 mol/L NaCl. Fractions of 1 mL were collected and assayed for absorbance at 280 nm and BP-53 by RIA. ALS was determined on 20-µL aliquots of peak B fractions by the routine assay method;

FIGURE 4 depicts the generation of the 150K complex from DEAE-Sephadex-fractionated serum. Peak A and B pools, as in Figure 3, were prepared by DEAE-Sephadex chromatography of 10 mL serum, then fractionated by Superose-12 chromatography. The samples, injected in a volume of 200 µL each, were peak A (a: 100 µL), peak B (b: 100 µL) peaks A and B (c: 100 µl each), mixed and incubated for 1 h at 22°C before loading, and whole serum (d: 33 µL). BP-53 immunoreactivity was measured on 50 µL of each 0.5 mL fraction. Arrows indicate 150K, 60K and 35K markers;

FIGURE 5 shows the comparison of BP-53 immunoreactivity and ALS activity, as indicated in

serum fractionated on Superose-12. Each fraction was 0.5 mL. The arrows indicate 150K, 60K and 35K markers. Note that the method used to detect ALS protein only detects protein not present as the 150K complex;

FIGURE 6 depicts acid lability of ALS activity. Samples of normal serum (a) or partially pure ALS (b) were diluted in ALS assay buffer and adjusted to the pH values shown with 1 mol/L HCl or NaOH. After 30 min at 22°C, the samples were reneutralized and assayed for ALS activity in the routine assay (10 µL serum or 600 ng ALS preparation/incubation);

FIGURE 7 shows the effect of IGFs on ALS binding to BP-53. Left panel: Increasing concentrations of BP-53 were incubated in a 300 µl reaction volume with [^{125}I]-labeled ALS tracer in the presence or absence of IGF-I or IGF-II (50 ng/tube), as indicated. Right panel: Competitive binding study in which 10 ng BP-53 plus 10 ng IGF-I or IGF-II was incubated in 300 µl with ALS tracer and increasing concentrations of unlabelled ALS. Tracer bound to BP-53 was immunoprecipitated with anti-BP-53 antiserum R-7.

FIGURE 8 shows the effect of ALS on IGF binding to BP-53. Left panel: Increasing concentrations of BP-53 were incubated in a 300 µl reaction volume with [^{125}I]-labeled IGF-I or IGF-II tracer (IGF-I* or IGF-II*) in the presence or absence of ALS (100 ng/tube), as indicated. Right panel: Competitive binding study in which 0.25 ng BP-53, in the presence or absence of 100 ng ALS, was incubated in 300 µl with IGF-II tracer and increasing concentrations of unlabelled IGF-I or IGF-II, as indicated. Tracer

bound to BP-53 was immunoprecipitated with anti-BP-53 antiserum R-7.

FIGURE 9 shows the effect of BP-53 and ALS on the gel chromatographic profile of [^{125}I]-labeled IGF-II tracer. Samples of 200 µl containing 50,000 cpm of IGF-II tracer, preincubated 2h at 22°C in the presence or absence of BP-53 (1 ng/200µl) or ALS (100 ng/200 µl), were chromatographed on a Superose-12 high performance chromatography column in 50 mM Na phosphate, 0.15 M NaCl, 0.02% Na azide, 0.1% bovine albumin, pH 6.5. Fractions of 0.5 ml were collected at 1 ml/min, and the radioactivity in each fraction determined. On each panel the three arrows indicate, from left to right, molecular weight markers of 150 kDa, 60 kDa and 7.5 kDa. Left panel: solid symbols, IGF-II tracer; open symbols, tracer plus ALS. Right panel: solid symbols, tracer plus BP-53; open symbols, tracer plus BP-53 plus ALS.

FIGURE 10 shows competition by increasing concentrations of acidified-neutralized human serum from normal, hypopituitary or acromegalic subjects in the routine ALS assay, in which 600 ng of partially purified ALS/250 µL incubation medium (i.e. 2.4 µg/mL) gave a 150K/60K ratio of approximately 1 in the absence of added serum. The serum concentration is expressed in terms of volume (a) or in terms of the immunoreactive BP-53 content (b). The acidified-neutralized serum samples illustrated contained 4.49 µg/mL (normal), 0.93 µg/mL (hypopituitary), or 10.49 µg/mL (acromegalic) BP 53 by RIA; and

FIGURE 11 depicts competition by pure BP-53 in the routine ALS assay, (a) the effect of increasing BP-53 concentrations after preincubation without IGFs

or with a 3.5-fold molar excess of pure human IGF-I or IGF-II, as indicated. Panel (b), shows the effect of a fixed BP-53 concentration (0.8 µg/mL) preincubated with increasing concentrations of IGF-I or IGF-II.

EXAMPLES

EXAMPLE 1

Materials:

Fresh human serum for ALS preparation was obtained from laboratory volunteers. Cohn Fraction IV of human plasma, provided by Commonwealth Serum Laboratories, Melbourne, Australia, was used as starting material to prepare human IGF-I and IGF-II, and the IGF-binding protein BP-53. DEAE-Sephadex A-50, SP-Sephadex C-25, electrophoresis standards, and the Superose 12 HR 10/30 column were obtained from Pharmacia, Sydney; Affi-Gel 10 and Affi-Gel 15 were purchased from Bio-Rad; and the PolyWAX LP (polyethleneimine) anion exchange HPLC column (200 x 4.6 mm) was from PolyLC, Columbia, MD. All other reagents were at least analytical grade.

Human IGF-I and IGF-II were isolated and iodinated as previously described (Baxter, R.C., and De Mellow, J.S.M. (1986) Clin. Endocrinol. 24, 267-278; and Baxter, R.C., and Brown, A.S. (1982) Clin. Chem. 28, 485), and IGF-I tracer was further purified by hydrophobic interaction chromatography (Baxter, R.C., and Brown, A.S. (1982) Clin. Chem. 28, 485). The 53K IGF-binding protein BP-53 was purified from Cohn fraction IV as previously described (Martin, J.L., and Baxter, R.C. (1986) J. Biol. Chem. 261, 8754-8760), and a covalent complex with [^{125}I]IGF-I, cross-linked using disuccinimidyl

suberate, was prepared and purified by gel chromatography according to the method of Baxter, R.C., and Martin, J.L. (1986) J. clin. Invest. 78, 1504-1512. A 28K IGF-binding protein BP-28 was purified from human amniotic fluid by affinity chromatography and reverse phase high pressure liquid chromatograph according to Baxter, R.C., Martin, J.L. and Wood, M.H. (1987), J. Clin. Endocrinol. Metab. 65, 423-431.

ALS Iodination and Radioimmunoassay:

[^{125}I]-labeled ALS was prepared by reacting 5 µg ALS in 50 µl M Na phosphate buffer, pH 7.4, for 20 sec with 1 mCi Na^{125}I and 10 µg chloramine-T, then terminating the reaction with 50 µg Na metabisulfite. An antiserum against ALS was raised by immunizing a rabbit over a 7-week period with 4 doses of approximately 100 µg purified ALS. Radioimmunoassay incubations in 0.5 ml final volume contained antiserum at 1:50,000 final dilution, [^{125}I]-labeled ALS (approx. 10,000 cpm per tube), and ALS in the range 0.5-100 ng/tube. After a 16 h incubation at 22°C, bound and free tracer were separated by centrifugation following the addition of goat anti-rabbit immunoglobulin (2 µl), carrier normal rabbit serum (0.5 µl), and, after 30 min, 1 ml 6% polythylene glycol in 0.15 M NaCl.

Assay for ALS:

The routine assay for ALS activity was developed, based on the conversion of a covalent BP-53-IGF-I complex of approximately 60K to 150K form in the presence of ALS.

Samples to be tested for ALS activity were diluted to 200 µL in buffer containing 50 mmol/L sodium phosphate, 0.15 mol/L NaCl, and 0.2 g/L sodium

azide, pH 6.5, with 10 g/L bovine albumin. Cross-linked BP-53-IGF-I tracer (~80,000 cpm; 4ng) was added in 50 µL of the same buffer. After 25-30 min. of incubation at 22°C, 200 µL of the mixture was applied using a V-7 injector valve (Pharmacia) to a Superose-12 gel permeation column eluting at 1.0 mL/min (~2 megapascal pressure) in assay buffer without albumin. The column was calibrated with rabbit immunoglobulin G (Pentex; ~150K), which eluted mainly in fractions 22-24, peaking in fraction 23; BP-53-IGF-I tracer (~60K), which eluted mainly in fractions 25-27, peaking in fraction 26; IGF-I tracer bound to BP-28 (~35K, peaking in fraction 28); and IGF-I tracer (7.5K, peaking in fraction 33). Plotted as log (molecular mass) vs. elution volume, these four markers yielded a linear calibration curve (not shown). As a quantitative index of ALS activity (i.e. the degree of conversion of BP-53 to the 150K complex), the total radioactivity in fractions 22-24 was divided by that in fractions 25-27 to give a 150K/60K ratio. The values of this ratio typically varied between 0.1 and 2.0. The coefficient of variation of the 150K/60K ratio, based on analysis of variance of eight duplicate measurements covering a wide range of values, was 3.2%. Because each chromatography run took 30 min., and the precision of the assay was high, each determination was generally performed singly within each experiment.

In the absence of ALS, the radioactivity was found predominantly in fractions 25-27, corresponding to a molecular mass of 60K, typically giving a 150K/60K ratio of 0.10 or lower. Increasing concentrations of ALS in the preincubation caused

increasing conversion of the 60K tracer to the 150K form (fractions 22-24), giving higher values for the 150K/60K ratio. Both IGF-I and IGF-II tracers, preincubated with pure BP-53 but not covalently cross-linked, could also be converted to 150K by incubation with ALS. Other IGF acid-stable binding proteins structurally related to BP-53 but of smaller size (such as those of 20, 24, 26, 30 and 47K) also participate in this reaction to form corresponding smaller complexes. Cross-linked BP-53-IGF-II tracer was not tested. A dose-response curve using the purified ALS preparation produced according to Example 2 was constructed using cross-linked BP-53-IGF-I tracer. A highly reproducible sigmoidal semilog plot was obtained, which could be used as a standard curve for quantitating the ALS in unknown samples (for example during purification). A similar result is obtained if the tracer complexed to ALS is precipitated with an anti-ALS antiserum instead of being fractionated on a Superose-12 column.

EXAMPLE 2

Purification of ALS:

Fresh human serum or Cohn Factor IV paste of human plasma were used as a source of ALS. Fresh human serum (100-130 ml) was dialyzed against 2 x 50 vol of 0.05 M Tris-HCl pH 8.2 at 2°C, then loaded onto a column of DEAE Sephadex A-50 (5 x 23 cm) equilibrated with dialysis buffer at 22°C. The column was washed with 2 liters of dialysis buffer, then with 2-2.5 liters of the same buffer containing 0.15 M NaCl. This step removed all of the immunoreactive BP-53 from the column. ALS was eluted by applying 1 liter of 0.05 M Tris-HCl, 0.6 M NaCl,

pH 8.2, pumping at 1 ml/min. Fractions of 10 ml were collected and assayed for ALS activity and absorbance at 280 nm. Active fractions were combined (approximately 140 ml total) and dialyzed at 2°C against 5 liters of 50 mM sodium phosphate, 0.02% Na azide, pH 6.5.

Where Cohn Factor IV is the source of ALS, the frozen paste (600g) was broken into small pieces and extracted for 16 h at 2°C by stirring with 3 liters 50 mM Tris-HCl, 0.15 M NaCl, 0.02% sodium azide, pH 8.2. The mixture was centrifuged 30min at 12000 rpm in the GSA rotor of a Sorvall RC5C centrifuge, yielding a turbid green-brown supernatant fraction (2.8 liter). This was divided into two equal portions and loaded by gravity feed onto two columns of DEAE Sephadex A-50 (5 x 22 cm) equilibrated with extraction buffer, and each column was washed with 2 liters of buffer. At this stage a predominant blue-green band was concentrated in the upper half of the column. Sometimes this band started to migrate through the column during the washing step; in these cases the washing volume was decreased to 1 liter. ALS was eluted from the column by a linear 0.15 - 0.35 M NaCl gradient in 50 mM Tris-HCl, 0.02% sodium azide, pH 8.2 (2 liter total volume). Fractions of 10 ml were collected and assayed for ALS activity and absorbance at 280 nm (or protein by a Biuret method). Active fractions from the two parallel columns were combined (approximately 1 liter total), diluted two-fold with 50 mM sodium phosphate pH 6.5, and the pH adjusted to 6.5 by slow addition of 1 M HCl. Since the active fractions corresponded closely with the blue-green protein in the eluted fractions, this provided a convenient visual marker for the

progress of the activity through the ion-exchange procedure.

The ALS containing fractions obtained from plasma or Cohn Factor IV, as detailed above, were applied to either one of two IGF affinity columns: (1) Affi-Gel 15 column (1 x 12 cm) to which 3 mg IGF-II had been coupled exactly as previously described (Martin, J.L., and Baxter, R.C. (1986) J. Biol. Chem. 261, 8754-8760), or (2) Affi-Gel 10 column (1 x 15 cm) to which a mixture containing approximately 5 mg IGF-I and 2 mg IGF-II had been coupled by the same procedure. The affinity column was loaded with BP-53, prepared exactly as previously described (Martin, J.L., and Baxter, R.C. (1986) Supra). Briefly, 600 g Cohn paste was homogenized with 5 vol of 2 M acetic acid, 75 mM NaCl, the mixture was centrifuged, and the supernatant was depleted of endogenous IGFs by stirring 2-3 days with approximately 400 ml packed volume of SP-Sephadex C-25 which had been equilibrated in the homogenizing buffer at pH 3.0. The mixture was centrifuged to remove the gel, and the supernatant was adjusted to pH 6.5 in two steps, as previously described (Martin, J.L., and Baxter, R.C. (1986) Supra). The pH 6.5 supernatant was then pumped at approximately 0.5 ml/min onto the affinity column, and the column was washed at 1-2 ml/min with 250 ml of 50 mM Na phosphate, 0.5 M NaCl, pH 6.5, and 100 ml of 50 mM Na phosphate, pH 6.5.

ALS containing fractions from DEAE-Sephadex chromatography were pumped at 0.1 - 0.15 ml/min onto the IGF affinity column loaded with BP-53. This typically resulted in the retention of over 90% of the ALS activity. The column was washed at 1 ml/min

with 150 ml of 50 mM Na phosphate, pH 6.5, and 50 ml mM NaCl, 5 mM Na phosphate pH 6.5, to 1 wer the buffering capacity of the column. ALS was eluted by applying 50 mM Tris-HCl, 0.3 M NaCl, pH 8.5 to the column at 0.5 ml/min. This is illustrated in Figure 1 which shows a plot of elution volume from the affinity column against ALS (µg/ml) and absorbance at 280nm. Fractions of 2 ml were collected in siliconized glass tubes and assayed for ALS activity.

SDS-PAGE (10%) of the immunopurified ALS, under reducing conditions, yielded a doublet of closely associated bands with an approximate molecular weight of 90K. The doublet may be due to varying glycosylation of ALS. No other bands were present, this indicating that the ALS was homogeneous.

As an optional final purification step affinity-purified ALS was fractionated by high-performance anion exchange chromatography. Sample loads of 0.5ml per run were applied to a PolyWAX high performance anion exchange column equilibrated at 1.5 ml/min in 0..05 M ammonium hydrogen carbonate (unadjusted pH = 7.8). The ALS was eluted by applying a linear salt gradient (Model 680 Gradient Controller, Waters, Milford, MA) from 0.05 M to 0.5 M ammonium hydrogen carbonate (pH adjusted) over 15 min at 1.5 ml/min. In some preparations a concave gradient was used (gradient #7, Model 680 Gradient Controller) over the same concentration range, with comparable results. Absorbance at 280 nm was monitored using a Waters Model 441 Absorbance Detector. Fractions of 0.75 ml were collected and assayed for ALS activity. A single major protein peak emerged from the column after 9-10 min elution at 1.5 ml/min when a linear

gradient was employed, or 11-12 min using a concave gradient. All of the detectable ALS activity, determined by RIA, was associated with this peak, with the recovery of applied activity estimated at over 75%, and a further increase in specific activity of 1.6-fold. The ALS activity was always associated with a single peak.

Figure 2 shows purified ALS after HPLC fractionation electrophoresed on a linear 10-15% polyacrylamide gel, under both reducing and non-reducing conditions. The preparation appeared as a doublet of apparent molecular mass 84 and 86 KDa under either non-reducing (left panel) or reducing conditions. Acidification of the protein (prepared by adjusting 35µg of ALS in 50µl 0.05M ammonium hydrogen carbonate to pH 3 with 20µl of 1M acetic acid, incubating 15 min. at 22°C, and neutralizing with 10µl of 2M Tris base), which results in a substantial loss of activity, had no effect on the protein's mobility on SDS-PAGE when run either non-reduced or reduced. However, treatment with N-glycanase (25µg ALS boiled in 40µl 0.5% SDS for 3 min., then diluted in 0.55 M Na phosphate, pH 8.6 and Nonidet P-40 to final concentrations of 0.2 M and 1.25% respectively; then N-glycanase (Genzyme Corp., Boston, MA.) was added to a final concentration of 60 units/ml, and the mixture incubated at 16h at 27°C) to remove N-linked carbohydrate resulted in a significant decrease in apparent molecular mass, to 80 kDA non-reduced (left panel) and 66 kDA (right panel). Notably, the protein migrated as a single band after deglycosylation with N-glycanase, suggesting that the doublet seen in the native preparation is due to at least two glycosylation

variants. Under reducing conditions, the deglycosylated preparation showed several bands in the range 50 - 60 kDa, suggesting that further deglycosylation might be possible.

Table 1 summarizes the results of a typical ALS purification, one of four performed on a similar scale and with similar results. Fractions eluted from the DEAE-Sephadex column by 0.05 M Tris-HCl, 0.6 M NaCl, pH 8.2 (DEAE eluate #2), contained over 60% of the applied ALS immunoreactivity and 13% of the total protein, whereas fractions eluted with buffer containing 0.15 M NaCl (DEAE eluate #1) contained only 15% of the ALS activity, but 79% of the protein. Further purification of DEAE eluate #2 fractions by affinity chromatography on a column of BP-53 non-covalently bound to agarose-IGF yielded a 200-fold increase in ALS specific activity.

The purification strategy employed was constrained by the fact that the sub-unit is irreversibly inactivated at low pH, but took advantage of the fact that it is reversibly dissociated from the BP-IGF complex at high pH. The key step in the purification is an unusual application of affinity chromatography in which the affinity ligand (BP-53) is not attached to the agarose matrix by a covalent bond, but appears to act as a non-covalent bridge between agarose-IGF beads and the ALS. In retrospect it is clear that the use of a covalent agarose-BP-53 matrix would not have worked, since BP-53 unoccupied IGF-I or IGF-II is unable to bind ALS. The optional final step, high performance chromatography on a PolyWAX (weak anion-exchange) column with salt gradient elution, essentially reiterates the initial step of

DEAE-Sephadex chromatography, but at much higher resolution.

EXAMPLE 3

Amino-terminal Sequence of ALS:

The N-terminal sequence of ALS was determined on an estimated 35 µl sample of HPLC purified material by Edman degradation using an Applied Biosystems 470A automatic gas-phase protein sequencer coupled to a 120A PTH Analyzer using a standard PTH program. Cys residues were confirmed on a second sample after reduction with mercaptoethanol and carboxymethylation with iodoacetic acid.

In two determinations, amino-terminal analysis showed approximately equimolar amounts of Gly and Ala for the first residue, despite the fact that the preparation analyzed was from the serum of a single donor. Analysis of the first 18 residues yielded the sequence Gly(Ala)-Asp-Pro-Gly-Thr-Pro-Gly-Glu-Ala-Glu-Gly-Pro-Ala-Cys-Pro-Ala-Ala-Cys-, the Cys residues in positions 14 and 18 being confirmed on a reduced and carboxymethylated sample. This amino acid sequence shows no obvious homology to other IGF proteins or receptors.

EXAMPLE 4

DEAE-Sephadex Fractionation of Serum:

The starting material was an ammonium sulfate fraction of serum from 30-50% saturation prepared according to previously published tables (Green, A.A., and Hughes, W.L., Methods of Enzymol. 1, 67). The resulting precipitate, dialyzed against an excess of 50 mmol/L Tris-HCl, pH 8.2, contained approximately 75% of the BP-53 immunoreactivity of

whole serum. In subsequent studies, the ammonium sulfate fractionation was found to be unnecessary, and whole serum dialyzed against Tris-HCl buffer was used. A 1 x 17.5 cm. column of DEAE-Sephadex A-50, equilibrated in 50 mmol/L Tris-HCl, pH 8.2, was loaded with a 1-mL dialyzed sample and eluted with 35 mL starting buffer, 50 mL starting buffer plus 0.15 mol/L NaCl, and 50 mL starting buffer plus 0.6 mol/L NaCl. In a larger scale protocol, 10-mL dialyzed samples were loaded onto a 1.5 x 20 cm column and eluted with 50, 100 and 100 mL, respectively, of the three buffers. The major protein peak eluting in the presence of 0.15 mol/L NaCl was termed peak A, and the peak emerging in 0.6 mol/L NaCl was termed peak B (Fig. 3).

The majority of immunoreactive BP-53 was found in the first peak (peak A), whereas the second peak (peak B) contained ALS activity with very little BP-53 immunoreactivity (Fig. 3 bottom). A small amount of ALS activity also was detected in fractions corresponding to the descending side of peak A (not shown). Similar results were obtained in six separate experiments.

Figure 4, representative of three similar experiments, shows the BP-53 immunoreactivity profiles for these protein peaks, separately and after preincubation together when fractionated by Superose-12 chromatography. The BP-53 immunoreactivity from peak A eluted primarily in a broad peak between fractions 25 and 30, corresponding to a molecular mass range of approximately 30-60K, with a small peak in fractions 22-24, corresponding to 150K (Fig. 4a). The barely detectable BP-53 activity from peak B eluted from Superose-12

predominantly in fractions 23-25 (Fig. 4b). After mixing peaks A and B and preincubating for 60 min at 22°C, over 50% of the peak A BP-53 activity had shifted from 30-60K to 150K, with the remainder still at 30-60K (Fig. 4c). This may be compared with the BP-53 profile in whole serum, in which over 90% of the activity was at 150K and only 5-10% in the 30-60K region (Fig. 4d). The ALS activity of peak B, as depicted in Fig. 4c, was unaffected by dialysis of peak B fractions against Tris buffer containing no NaCl or 0.6 mol/L NaCl, indicating that neither high salt nor any other dialyzable molecule was involved in the reaction between BP-53 and the ALS in peak B.

Superose-12 Fractionation of Serum:

Serum from normal subjects was diluted 1:1 with 50 mmol/L sodium phosphate, 0.15 mol/L NaCl, and 0.2 g/L sodium azide, pH 6.5, and 200 µL was applied to the Superose-12 column and eluted as described for the routine ALS assay. Each fraction was then tested for BP-53 and ALS activity.

As shown previously in Figure 4d, BP-53 immunoreactivity peaked in fraction 23, corresponding to 150K (Fig. 5). In contrast, in three experiments the peak ALS activity reproducibly eluted in fraction 24 (Figure 5), corresponding to 90-110K, suggesting that there is an excess of ALS over BP-53 in serum and that the free sub-unit has an apparent molecular mass of 90-110K. A similar peak of ALS activity was found in serum from which more than 99% of immunoreactive BP-53 (i.e. essentially all of the 150K complex) had been removed by affinity chromatography on a column of anti-BP-53 antibody coupled to agarose (not shown), confirming that the ALS detectable at 90-110K was not complexed to

BP-53. A comparable result was found when serum was fractionated by ion exchange chromatography, as shown in Fig 3 and peak B was subjected to Superose-12 chromatography.

Increasing volumes of serum when tested in the routine ALS assay, gave a dose dependent increase in the 150K/60K ratio (not shown). The ALS detectable in whole serum appeared to be GH dependent, as higher activity was found in serum from five acromegalic subjects and lower activity in serum from five GH-deficient subjects than was detectable in samples from normal subjects. This GH-dependence provides the basis for a diagnostic assay for determining GH levels in serum, and may be exploited in the diagnosis of growth disorders using, for example, antibodies directed against ALS.

EXAMPLE 5

Acid lability of ALS:

The acid lability of purified ALS or ALS in whole serum (following procedure of Example 2) was evident by its irreversible inactivation on exposure to low pH. The protein appeared quite stable at pH values as low as 5, but below this it rapidly lost activity (Fig. 7); and the 150K/60K ratio decreased by over 80% at pH 3. This decrease in the 150K/60K ratio is equivalent to a decrease in apparent ALS activity of over 99%. In contrast, exposure at high pH values (up to pH 10) had no effect on ALS activity in whole serum or the purified preparation.

EXAMPLE 6

Functional Studies:

To determine the binding kinetics of ALS to

BP-53, incubations were set up containing [^{125}I]-labeled ALS and various concentrations of BP-53 and IGF-I or IGF-II. Complexes of ALS tracer with BP-53 were detected after immunoprecipitation using an antiserum against BP-53 which has previously been shown to react with the BP in both free and complexed forms (Baxter, R.C. and Martin, J.L. (1986) J. Clin. Invest. 78, 1504-1512). Figure 7 (left) shows the effect of increasing BP-53 concentrations, over the range 0.25 to 100 ng/tube (0.016 to 6.3 nM), on complex formation. In the absence of IGF-I or IGF-II, there was little or no reaction between ALS tracer and BP-53. In the presence of a molar excess of IGF-I or IGF-II (50 ng/tube or 22 nM), a dose-dependent increase in ALS tracer binding was seen, increasing to 50% specific binding to 100 ng/tube of BP-53. Higher concentrations of BP-53 could not be tested due to limitations of the immunoprecipitation system. Complex formation was consistently higher in the presence of IGF-I than IGF-II.

The binding affinity between ALS and BP-IGF complexes was estimated from competitive binding studies. As shown for a typical experiment in Fig. 7 (right), binding of [^{125}I]-labeled ALS was again greater in the presence of IGF-I than IGF-II. In three similar experiments, the mean specific binding (± SEM) to 10 ng/tube BP-53 (i.e. corrected for radioactivity precipitated in the absence of BP-53) was 24.3 ± 4.4% in the presence of excess IGF-I, and 19.6 ± 3.9% in the presence of excess IGF-II ($P = 0.009$ by paired t-test). Increasing concentrations of unlabelled ALS caused a dose-dependent displacement of [^{125}I]-labeled ALS from

immunoprecipitatable complexes. Analysis of binding data by Scatchard plot revealed a nonspecific binding component (association constant $<10^6$ M^{-1}) and a single specific binding component with a slightly higher affinity for BP-IGF-I than BP-IGF-II. In three similar experiments the mean association constant (± SEM) for ALS binding to BP-IGF-I was 6.06 ± 0.71 x 10^8 M^{-1}, and for ALS binding to BP-IGF-II, 4.12 ± 0.29 x 10^8 M^{-1}. The binding site concentration was 1.28 ± 0.46 mol ALS/mol BP-53 in the present of IGF-I, and 1.18 ± 0.29 mol/mol in the presence of IGF-II, assuming the molecular masses of ALS and BP-53 are 86 kDa and 53 kDa respectively. If the calculation is based on the reduced molecular mass of 43 kDa for BP-53, the binding site concentrations are 1.04 ± 0.37 mol/mol and 0.96 ± 0.33 mol/mol respectively. This result is consistent with a single binding site for ALS per molecule of BP-53.

The lack of effect of ALS on the interaction between BP-53 and the IGFs is shown in Fig. 8. [^{125}I]-labeled IGF-II consistently showed higher binding to increasing concentrations of BP-53 than [^{125}I]-labeled IGF-I. The binding of either tracer was unaffected by the addition of 100 ng pure ALS per tube (Fig. 8, left). Competitive binding curves for the displacement of [^{125}I]-labeled IGF-II from BP-53 by increasing concentrations of unlabelled IGF-I and IGF-II are shown in Fig. 8 (right). IGF-II was consistently more potent than IGF-I in displacing tracer from BP-53, and neither displacement curve was affected by the addition of 100 ng/tube of ALS. Similar results were seen when [^{125}I]-labeled IGF-I was used as tracer (not shown).

To confirm that pure ALS was capable of

converting the BP-53 to the 150 kDa form, incubation mixtures similar to those used in the competitive binding experiments shown in Fig. 8 were fractionated by gel chromatography on Superose 12. [^{125}I]-labeled IGF-II appeared as a single peak of radioactivity, peaking in Fraction 34. Incubation of this tracer with pure ALS (100 ng/200 µl) before fractionation had no effect on the radioactive profile, indicating that ALS alone was unable to bind IGF-II tracer (Fig. 9, left). Incubation of IGF-II tracer with 1 ng/200 µl pure BP-53 resulted in the conversion of 70% of the radioactivity to a 60 kDa form, i.e. BP-53 - IGF-I. When this incubation also included 100 ng/200 µl pure ALS, the 60 kDa complex was substantially converted to a 150 kDa form (Fig. 4, right), demonstrating that complex formation required no components other than pure IGF, pure BP-53, and pure ALS.

EXAMPLE 7

Inhibition of ALS Binding to BP-53-IGF-I:

Various substances were tested for their ability to inhibit tracer BP-53-IGF-I binding to ALS. Human serum, when acidified and reneutralized to destroy its endogenous ALS activity and leave its acid-stable BP-53 intact, contained potent competing activity. On comparing samples from normal, acromegalic, and GH-deficient subjects in this way in three separate experiments, the competing activity showed strong GH-dependence, as expected for the endogenous BP-53 in such samples. This is illustrated for one such experiment in Figure 10a. When the curves in Figure 8a were replotted in terms of the immunoreactive BP-53 content of each sample, they became

superimposable (Figure 10b), indicating that the endogenous BP-53 in acidified whole serum could compete with cross-linked tracer in the ALS reaction. Under the conditions used in this assay, approximately 1 µg BP-53/mL reaction volume (i.e. 250 ng/250 µL) fully displaced cross-linked tracer from the BP-ALS complex, with half-maximal displacement at 200-250 ng/mL BP-53.

In contrast to the endogenous BP-53 in acidified serum, pure BP-53, when tested at up to 0.8 µg/mL, was unable to compete with cross-linked tracer in the ALS reaction (Figure 11). However, after preincubation for 30 min at 22°C with a 3.5-fold molar excess of pure human IGF-I or IGF-II (i.e. 500 ng IGF/µg BP-53), purified BP-53 could fully displace cross-linked tracer from the BP-53-ALS complex. Also tested, and found not to compete in the ALS reaction, were the following; purified amniotic fluid BP-28 (0.8 µg/mL), BP-28 preincubated with excess IGF-I or IGF-II (0.5 µg/mL), or human GH (20 µg/mL). These experiments again indicate that only BP-53 that is occupied by IGF-I or IGF-II can take part in the reaction with ALS and strongly suggest that BP-28, whether occupied or not, is unable to react with ALS.

The scientific articles previously referred to are incorporated herein in their entirety.

The claims form part of the description.

TABLE 1

Purification of the acid-labile subunit from human serum

The purification steps were as described in Example 2. ALS, determined by radioimmunoassay, is expressed in terms of a pure standard preparation. DEAE eluate #1 refers to the pool of fractions eluted from DEAE-Sephadex by buffer containing 0.15 M NaCl. DEAE eluate #2 refers to the pool of fractions eluted by buffer containing 0.5 M NaCl; this pool was dialyzed before assay. Affinity eluate is the pool of fractions eluted from the affinity column, then concentrated by ultrafiltration. HPLC pool is the pool of active fractions recovered from the HPLC step.

Purification Step	Volume	Total Protein	Total ALS	ALS specific Activity	Purification Factor	Recovery
	ml	mg	µg	µg/mg protein	fold	%
Serum	120	11860	8290	0.70	1.0	100
DEAE eluate #1	2530	9360	1280	0.14	0.2	15.4
DEAE eluate #2	132	1530	5060	3.31	4.7	61.0
Affinity eluate	4.5	2.61	1850	709	1010	22.3
HPLC pool	15.75	1.22	1400	1148	1640	16.9

CLAIMS:

1. The acid-labile sub-unit (ALS) of insulin like growth factor binding protein complex in biologically pure form.

2. A composition of matter consisting essentially of the acid-labile sub-unit (ALS) of the insulin like growth factor binding complex.

3. ALS according to claim 1 or 2 which has a molecular weight of approximately 80-115 kilodaltons as determined by reducing SDS-polyacrylamide gel electrophoresis.

4. ALS according to any one of claims 1 to 3 having the following partial N-terminal amino acid sequence:

Gly
AspProGlyThrProGlyGluAlaGluGlyProAlaCysProAlaAlaCys-Ala

wherein said first amino acid may be Gly or Ala.

5. A method for the preparation of the in-vivo IGF binding protein complex comprising the step of admixing together IGF, the acid-stable IGF binding protein (BP 53) and ALS.

6. A method according to claim 5, wherein the IGF, acid-stable IGF binding protein (BP 53) and ALS are biologically pure.

7. A composition containing the in-vivo IGF protein complex, prepared according to claim 5 or 6,

in association with a pharmaceutically or veterinarially acceptable carrier or excipient.

8. A method for the treatment of wounds comprising applying to the wound or the area adjacent the wound, or administering to the wounded patient, a therapeutically effective amount of a composition as claimed in claim 7.

9. A method for promoting cellular growth in animals and humans comprising administering to a patient a therapeutically effective amount of a composition as claimed in claim 7.

10. A method for the preparation of ALS which comprises the steps of:

(a) applying a source of ALS to a support matrix having attached thereto IGF bound to or associated with the acid-stable IGF binding protein, whereby the ALS in the applied material binds to the acid stable binding protein and non-bound material is separated from the support matrix; and

(b) selectively eluting and recovering the ALS protein from the IGF protein complex.

11. A method for the preparation of ALS which comprises the steps of:

(a) binding IGF to a support matrix;

(b) adding the acid-stable IGF binding protein to the support matrix such that it binds to the IGF;

(c) applying a source of ALS to the support matrix whereby the ALS in the applied material binds to the acid stable binding protein and non-bound material is separated from the support matrix; and

(d) selectively eluting the ALS protein from the IGF protein complex.

12. A method according to claim 10 or 11, wherein the source of ALS is human plasma or Cohn Fraction IV of human plasma.

13. A method according to claim 10 or 11, wherein the source ALS applied to the support matrix is partially purified.

14. A method according to claim 13, wherein the source of ALS is partially purified by fractionation on an ionic resin.

15. A method according to claim 14, wherein the ionic resin is a cation exchange resin.

16. A method according to any one of claims 11 to 15, wherein the eluted ALS from step (d) is further fractionated by HPLC or FPLC.

17. ALS when prepared according to the method of any one of claims 10 to 16.

18. A method for detecting ALS in a biological fluid comprising the steps of:

(a) binding proteins of a biological fluid to a support matrix;

(b) reacting the support matrix with antibodies directed against ALS; and

(c) detecting antibody binding.

19. A method as claimed in claim 18, wherein the

biological fluid is serum.

20. A method for detecting the levels of ALS in a body fluid, which comprises fractionating the body fluid on a size fractionation matrix to separate free ALS from the other components of the insulin growth factor binding complex, and thereafter quantitating the levels of ALS in the fractionated sample.

21. A method according to claim 20, wherein the levels of ALS in a body sample are detected by measuring the extent of formation of a reconstituted insulin like growth factor binding protein complex.

22. A method according to claim 20 wherein levels of ALS are quantitated by determining the extent of binding of anti-ALS antibodies to ALS in the fractionated sample.

23. A recombinant nucleic acid sequence encoding the acid-labile sub-unit (ALS) of insulin like growth factor.

24. A recombinant nucleic acid sequence according to claim 23, which encodes ALS, said ALS having the following partial N-terminal amino acid sequence:

Gly
AspProGlyThrProGlyGluAlaGluGlyProAlaCysProAlaAlaCys-Ala

wherein the first amino acid may be Gly or Ala.

25. An expression vector containing the DNA

sequence of claim 23.

26. A host cell, which is a procaryotic or eukaryotic cell, transformed with the expression vector of claim 25.

27. The recombinant nucleic acid sequence of claim 23 which is cDNA.

28. ALS when produced by the host cell of claim 26.

29. An antibody reagent capable of binding to ALS.

30. An antibody reagent according to claim 29 which is a monoclonal or polyclonal antibody.

31. An antibody reagent according to claim 29 or 30 which is labelled with one or more reporter groups.

32. An antibody reagent according to claim 31, wherein the reporter group is selected from fluorescent groups, enzymes or colloidal groups.

33. A nucleic acid isolate comprising nucleic acid encoding the sequence:

Gly
AspProGlyThrProGlyGluAlaGluGlyProAlaCysProAlaAlaCys-
Ala

wherein the first amino acid may be Gly or Ala.

34. A nucleic acid isolate comprising nucleic

acid encoding residues 1-5, 2-7, 5-9, 7-11, 8-14, 11-15, 13-17, 3-9, 2-8, 4-10, 6-12, 8-14, 10-16, 12-18, 1-6, 3-9, 5-11, 7-13, 9-15, 11-17, 4-9, 6-11, 8-13, 10-15, or 12-17 of ALS.

35. The nucleic acid isolate of claim 33 or 34 further comprising a replicable vector.

36. The nucleic acid isolate of claim 33 further comprising nucleic acid encoding a secretion signal sequence ligated to the 5' end of nucleic acid encoding the sequence:

Gly
AspProGlyThrProGlyGluAlaGluGlyProAlaCysProAlaAlaCys-Ala

wherein the first amino acid may be Gly or Ala.

37. A host cell transformed with the isolate of any one of claims 33 to 36.

38. A polypeptide comprising a fragment of ALS comprising a sequence of residues 1-5, 2-7, 5-9, 7-11, 8-14, 11-15, 13-17, 3-9, 2-8, 4-10, 6-12, 8-14, 10-16, 12-18, 1-6, 3-9, 5-11, 7-13, 9-15, 11-17, 4-9, 6-11, 8-13, 10-15, or 12-17 of ALS.

FIGURE 1

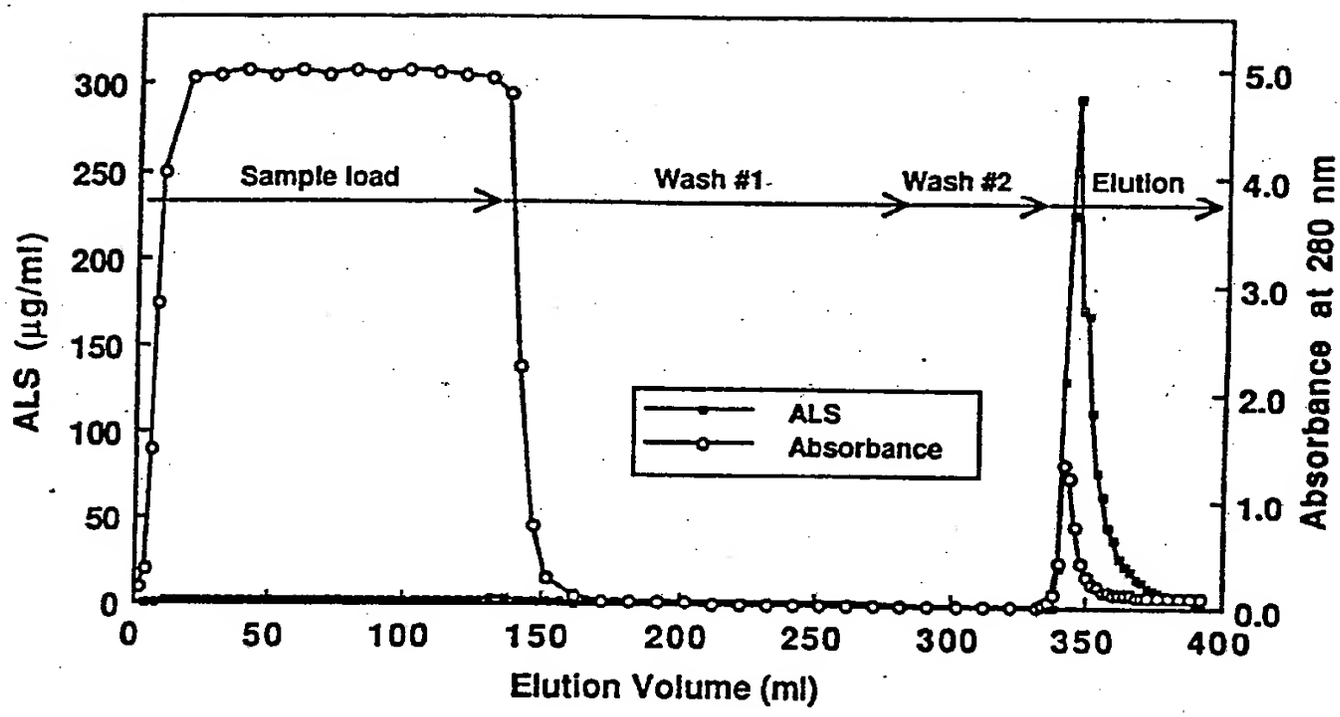

Sample load
Wash #1
Wash #2
Elution
ALS
Absorbance
ALS (µg/ml)
Absorbance at 280 nm
Elution Volume (ml)
0
50
100
150
200
250
300
350
400
0.0
1.0
2.0
3.0
4.0
5.0

FIGURE 2

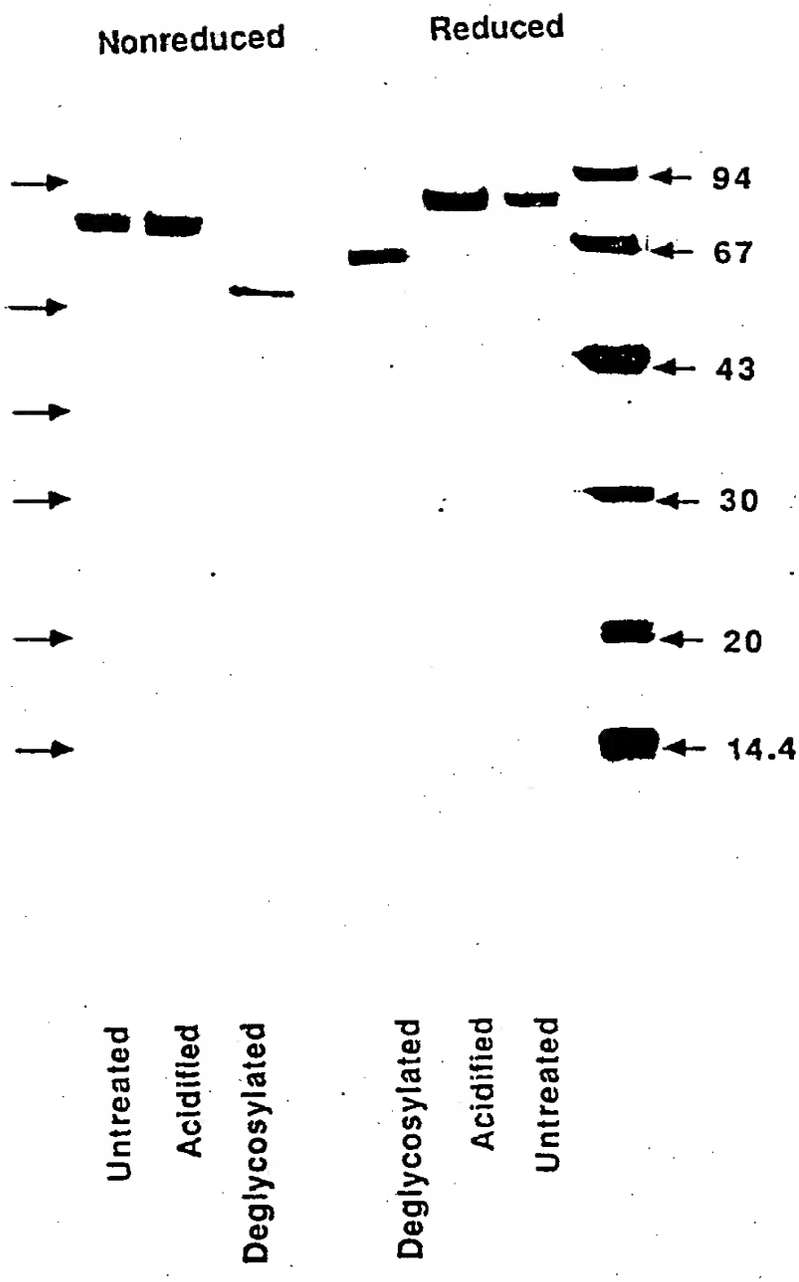

Nonreduced
Reduced
94
67
43
30
20
14.4
Untreated
Acidified
Deglycosylated
Deglycosylated
Acidified
Untreated

FIGURE 3

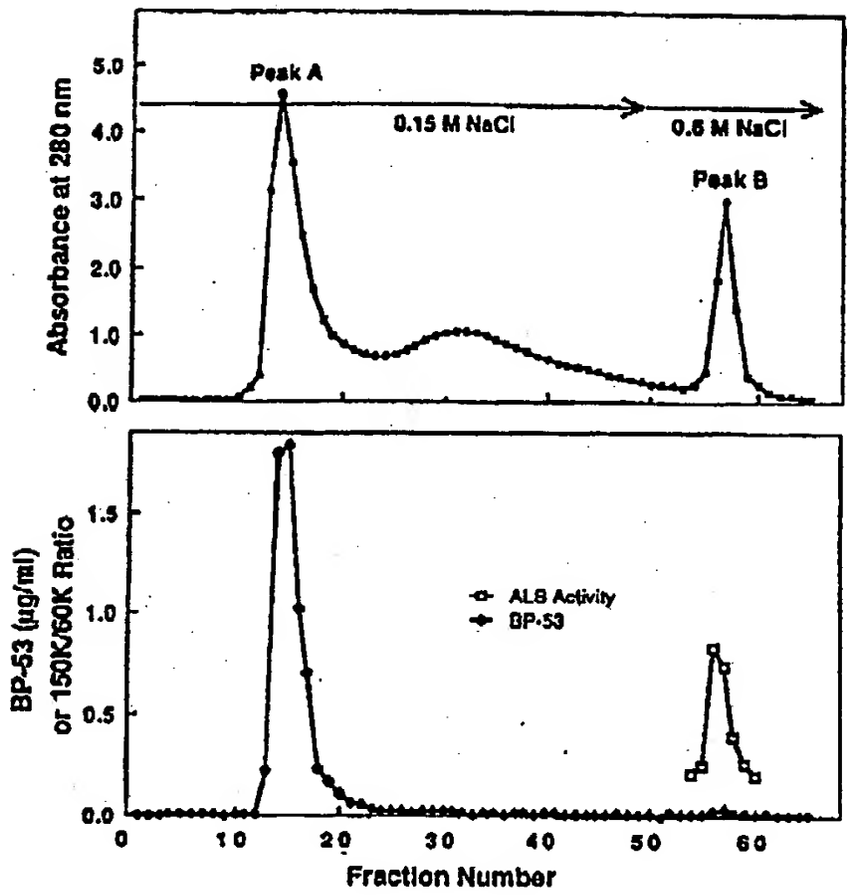

Peak A
0.15 M NaCl
0.6 M NaCl
Peak B
Absorbance at 280 nm
5.0
4.0
3.0
2.0
1.0
0.0
ALB Activity
BP-53
BP-53 (µg/ml)
or 150K/60K Ratio
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
Fraction Number

FIGURE 4

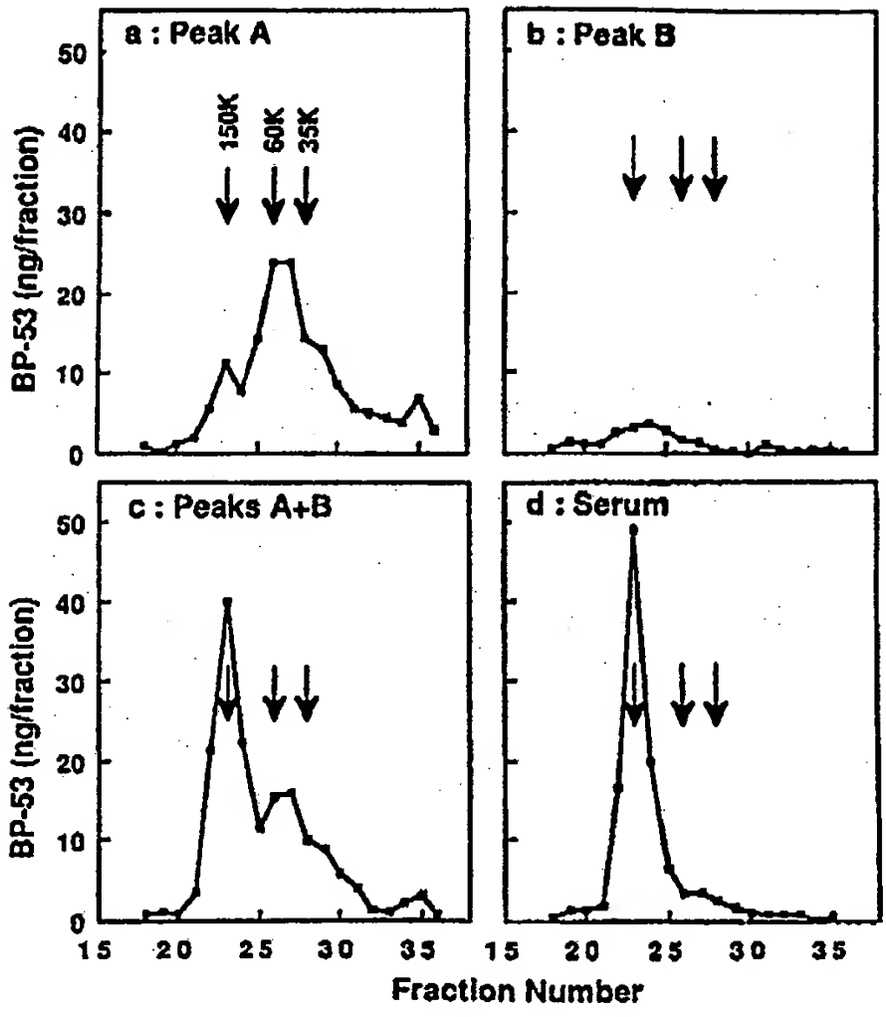


FIGURE 5

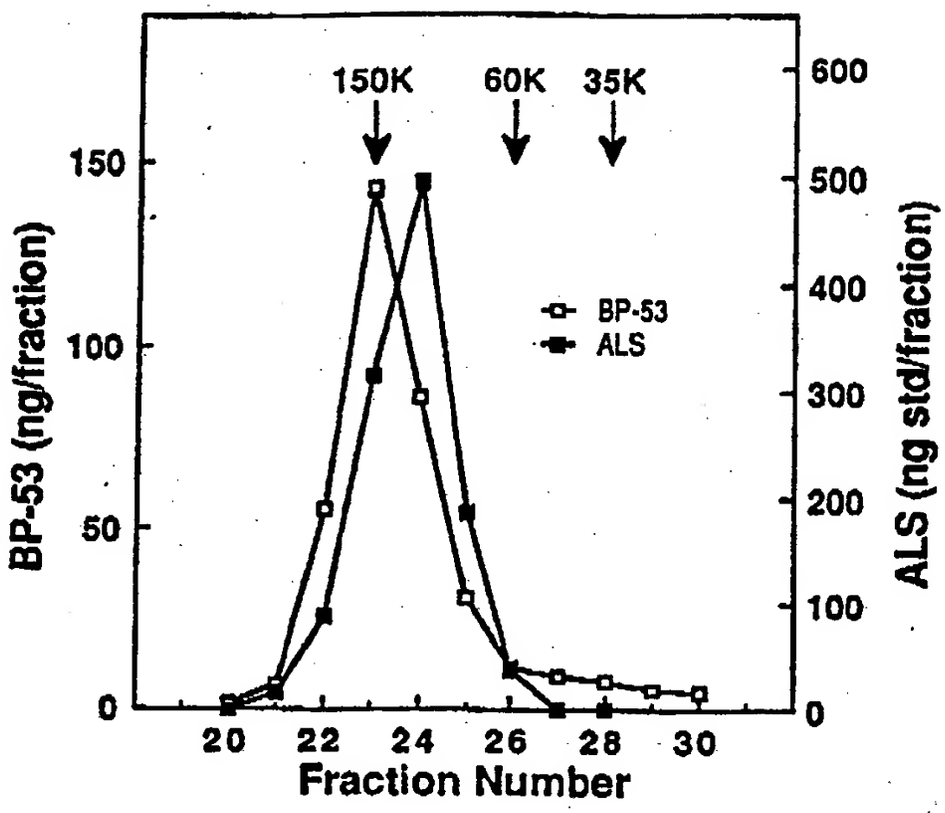


FIGURE 6

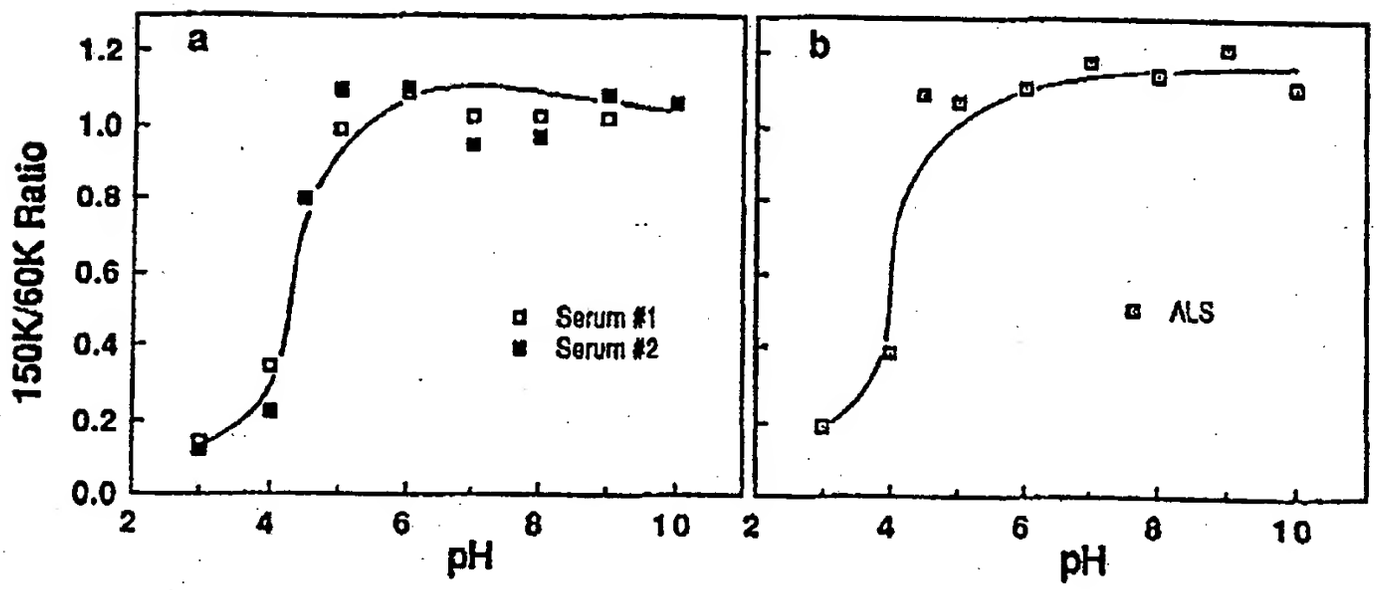


FIGURE 7

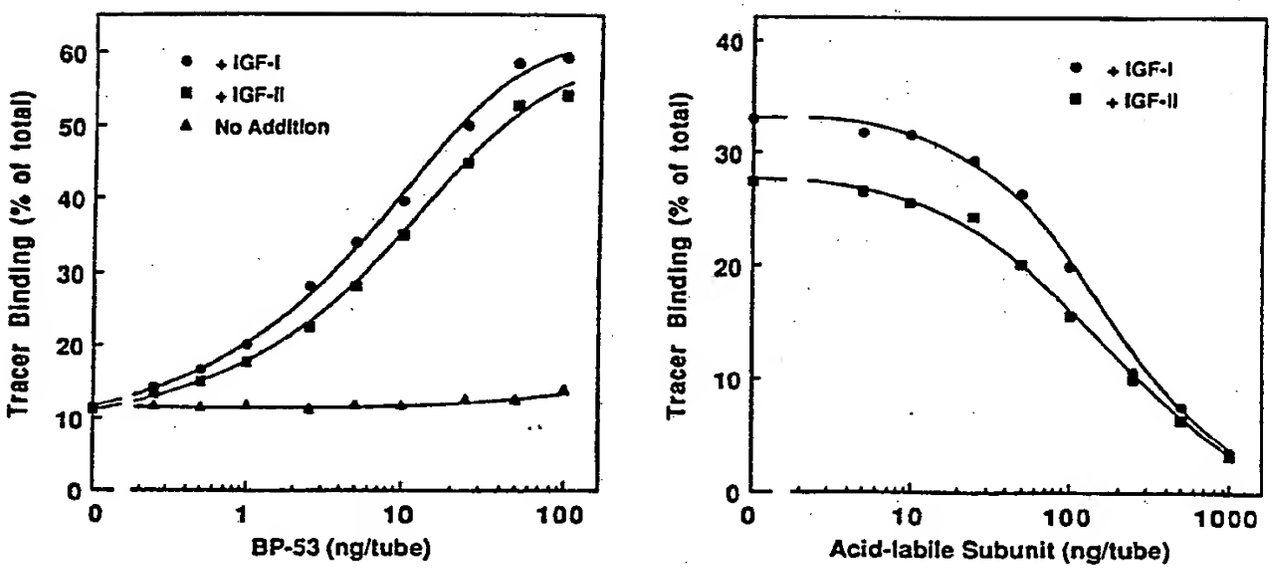

+ IGF-I
+ IGF-II
No Addition
Tracer Binding (% of total)
BP-53 (ng/tube)
0
10
20
30
40
50
60
0
1
10
100
+ IGF-I
+ IGF-II
Tracer Binding (% of total)
Acid-labile Subunit (ng/tube)
0
10
20
30
40
0
10
100
1000

FIGURE 8

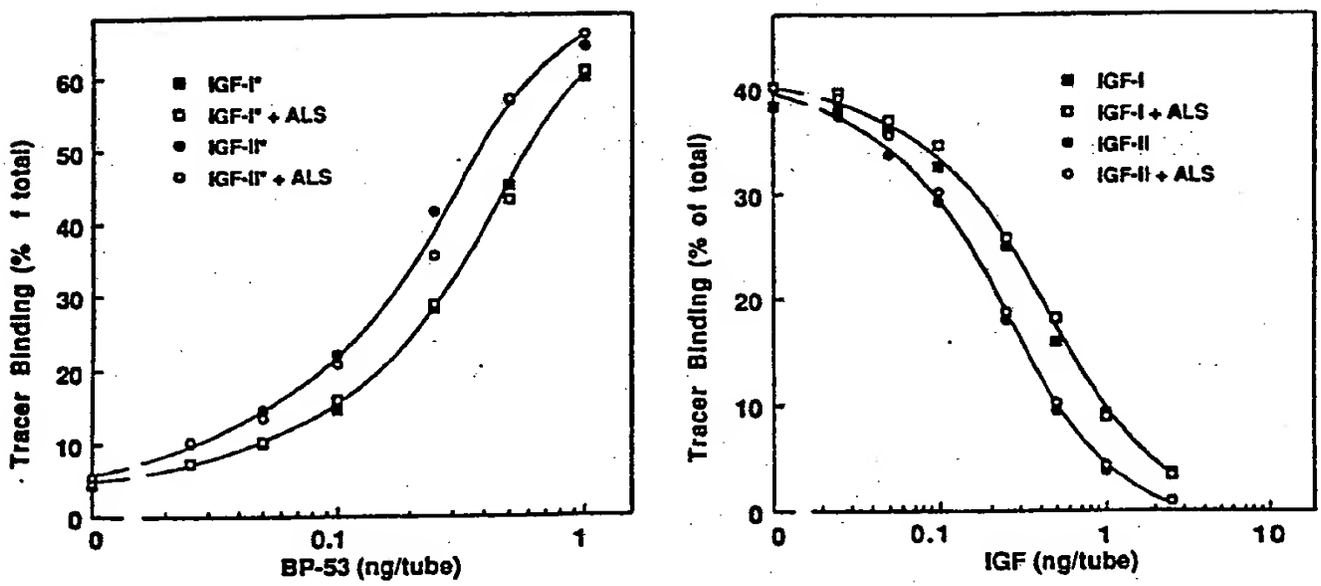

Tracer Binding (% f total)
IGF-I*
IGF-I* + ALS
IGF-II*
IGF-II* + ALS
BP-53 (ng/tube)
Tracer Binding (% of total)
IGF-I
IGF-I + ALS
IGF-II
IGF-II + ALS
IGF (ng/tube)

FIGURE 9

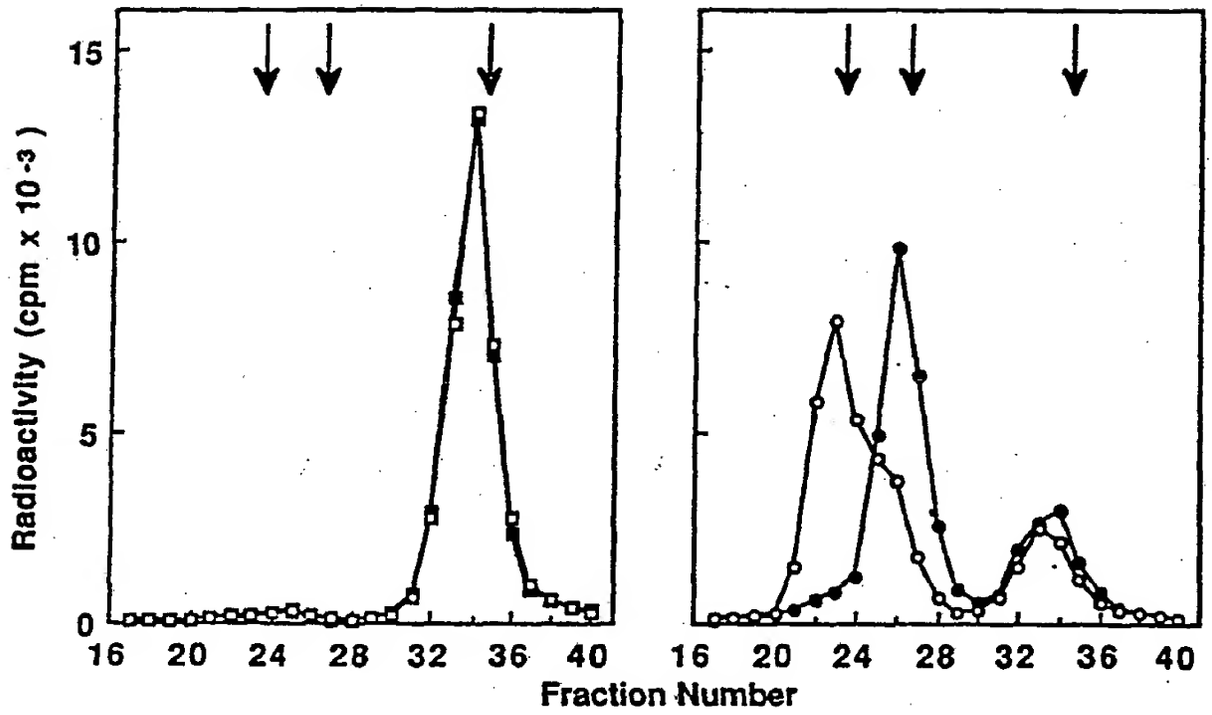

Radioactivity (cpm x 10-3)
15
10
5
0
16 20 24 28 32 36 40
16 20 24 28 32 36 40
Fraction Number

FIGURE 10

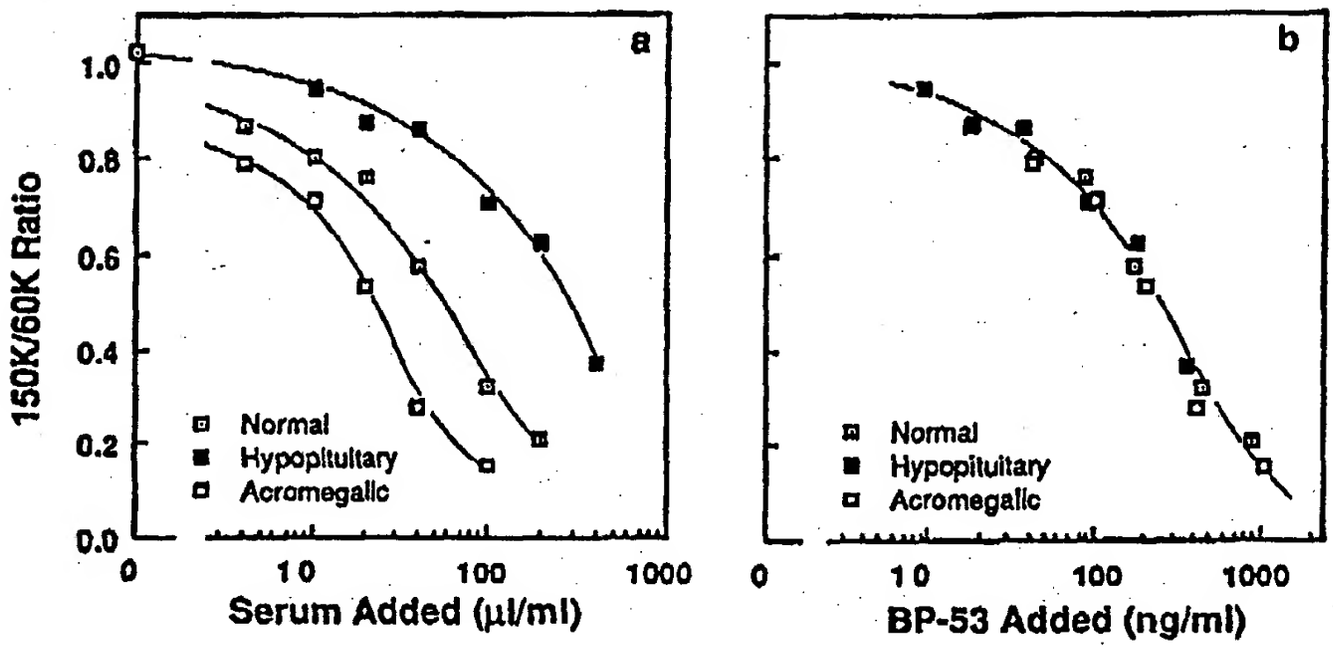
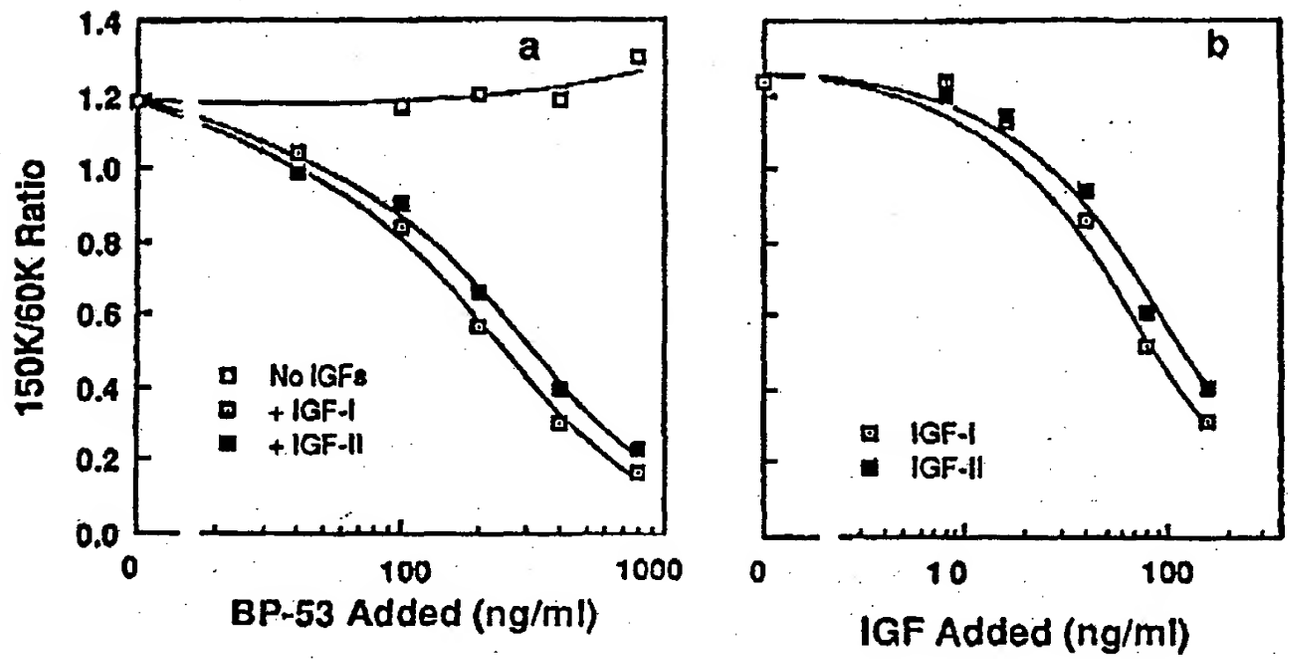


FIGURE 11



INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 89/00299

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C07K 015/12, 015/14, 007/06, G01N 033/74, C12N 015/00, C07H 021/04, C12Q 001/68, A61K 37/02, 37/36

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	WPI, WPIL, USPA, Derwent database - keywords: Insulin-like Growth Factor or Somatomedin or IGF

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AU: C07K 015/12 C07G 007/00 C07G 015/00 Chemical Abstracts keywords: as above, plus subunit

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X	Journal of Clinical Endocrinology and Metabolism. Vol 67 (2) 1988 pp265-272. Robert C Baxter. "Characterization of the Acid-Labile Subunit of the Growth Hormones Dependent Insulin-like Growth Factor Binding Protein Complex." (whole document)	1-7, 10-19, 29-32
X	Journal of Clinical Endocrinology and Metabolism Vol 51 (1) 1980 pp12-19. Richard W Furlanetto. "The Somatomedin C Binding Protein: Evidence for a Heterologous Subunit Structure" (See especially pp15-16)	1-2, 5
P,A	AU,A, 17020/88 (BIOGROWTH INC) 20 October 1988 (20.10.88)	

CONTINUED

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 October 1989 (19.10.89)	24 October 1989
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	J H CHAN

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	Endocrinology Vol 104 (2) 1978 p536-546 Moses, A.C. et al "Further Characterization of Growth hormone-Dependent Somatomedin-Binding Proteins in Rat Serum and Demonstration by Somatomedin-Binding Proteins Produced by Rat Liver Cells in Culture"	
A	Journal of Clinical Investigations Vol 75 (4) 1985 p1350-8 Wilkins, J.R. et al., "Affinity-labelled Plasma Somatomedin-C/ Insulin like Growth Factor Binding Proteins"	
A	Federation Proceedings Vol 41 (11) 1982 pages 2719-23 Czech, M.P. et al "Subunit structure and dynamics of the insulin receptor"	

CONTINUED

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.[X] Claim numbers 8-9,18-22, because they relate to subject matter not required to be searched by this Authority, namely:
Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods (Rule 39iv)

2.[] Claim numbers ..., because they relate to parts of the international application that do comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.[] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [X] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:
See Attached page

1.[]As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [X] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
Claims 1-22, 29-32 excluding claims 8,9,18,19,20,21 and 22 (Rule 39iv)

4. [] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest
[] The additional search fees were accompanied by applicant's protest.
[] No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	The Journal of Biological Chemistry Vol 257 (9) 1982 pages 5038-45 Massague J. et al "The Subunit Structures of Two Distinct Receptions for Insulin-like Growth Factors I and II and their Relationship to the Insulin Receptor"	
A	Biochemical and Biophysical Research Communications Vol 136 (1) 1986 pages 45-50 Pilch P.F. et al "The ligand binding subunit of the insulin-like growth factor I receptor has properties of a peripheral membrane protein"	

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING (contd)

Group 1 — Claims 1-22 construed in light of the description especially the examples are directed to an acid labile subunit (ALS) of insulin-like growth factor (IGF), isolated from natural sources using protein purification techniques and also a complex of ALS with other IGF proteins.

Claims 29-32 - are directed to an antibody reagent which binds to ALS. (Note: This group includes claims 8,9,18,19,20,21 and 22 whose subject matters are not required to be searched Rule 39iv.)

Group 2 — Claims 23-28, 33, 36 and 37 are directed to recombinant sequence encoding ALS, expression vectors, host cells and the expression of ALS therein. (Note: The description has not exemplified any such recombinant product.)

Group 3 — Claims 34 and 35 are directed to a probe which shows no similarity with cDNA of ALS.

Group 4 — Claim 38 is directed to a peptide, which consists of fragments of ALS, 4-7 amino acids in length, but shows no similarity with the biological activity of ALS.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 89/00299

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members			
AU 17020/88	DK 6776/88	EP 294021	EP 308500	
	IL 85983	WO 8807863	ZA 8802407	

END OF ANNEX